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Ultrasound Facilitated Marking of Gastrointestinal Tissue with Fluorescent Material

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***Abstract*— The epithelial lining of the gastrointestinal (GI) mucosal layer is an effective barrier to the contents of the gut lumen. Selective channels and tight junctions prevent contamination of the sterile internal environment of the body. Conversely, the gut barrier also prevents desired agents from entering the GI tissue. This hinders marking of tissue for further clinical follow-up. Focused ultrasound (US) may provide a potential means of overcoming the gut barrier and allowing penetration of material beyond it which was explored in a series of tests. Experiments were carried out on 14 individual postmortem-obtained murine small bowel samples for a total of 23 sonication/control paired tests. A favourable result of 80% indicated that focused US can pass a nanoscale fluorescent agent through the gut barrier. Further work is required to elucidate where the agent resides, intercellular or intracellular, post-sonication.**

Keywords— Gastrointetinal tract, Gut Barrier, Focused Ultrasound, Quantum Dot, Tissue Marking

I. INTRODUCTION

The gastrointestinal (GI) tract is a long hollow tube that extends from the mouth to the anus. As the mouth is a portal to substance ingress, beneficial or otherwise, the entire length of the bowel is considered continuous with the external environment. As the lumen is in contact with the exterior environment, the gut must form a barrier between the sterile interior and potentially compromised luminal contents, in addition to its digestive and absorptive functions. Barrier formation is a function of the mucosa or epithelial enterocytes which form a selective boundary to material, whether harmful or beneficial.

Fig. 1 illustrates the basic histological structure of the GI tract with a focus on how the uppermost layer forms a physical border to the external environment [1]. Firstly, the channels allowing the passage of specific molecules from the lumen into the enterocyte are discriminating in what material may pass. Secondly, the simple (mono)layer of columnar enterocytes bind tightly to one another via tight junctions, creating a barrier to agents passing between cells. This arrangement forms a relatively impermeable and selective barrier against luminal contents. In addition to these barriers, the gut mucosa has other protective mechanisms such as a mucous layer and continual enterocyte turnover preventing intracellular accumulation of undesirable material. The effectiveness of this barrier system can be measured down to the nanoscale [2].

In this context, the gut barrier system presents a challenge in regards to marking lesions identified in the mucosa for clinical follow-up. Because of the aforementioned defence mechanisms, passive marking of tissue can be ruled out, thus requiring a more active means of in-situ tissue labelling. Therefore, we have investigated the ability of focused US to overcome the gut barrier system and implant a marking agent into gut tissue actively. Focused US produces a variety of bioeffects which require relatively high intensities, but below those commonly taken to represent high intensity focused ultrasound (HIFU), including cavitation, acoustic radiation forces and mild-hyperthermia. Cavitation is the rapid expansion and contraction of gas pockets in an acoustic field. These expansions and contractions can lead to bubble implosion that can produce shockwaves, which have been shown to increase membrane permeability in sonication applications [3]. Acoustic radiation force (ARF) also has clinically useful effects. It has been shown that ARF has the ability to push agents towards a target location [4] and, in some cases, this can push the agents through cell membranes [5].

The purpose of the experiment reported here was to test the ability of focused US to overcome the gut barrier system and actively implant an agent into gut tissue

II. *MATERIALS AND METHODS*

A. Mouse Bowel

Sonication experiments were performed on 14 wild type (WT) H2B(gfp) mice. Table 1 outlines the sample demographics including age (range [days: 53-115, mean:

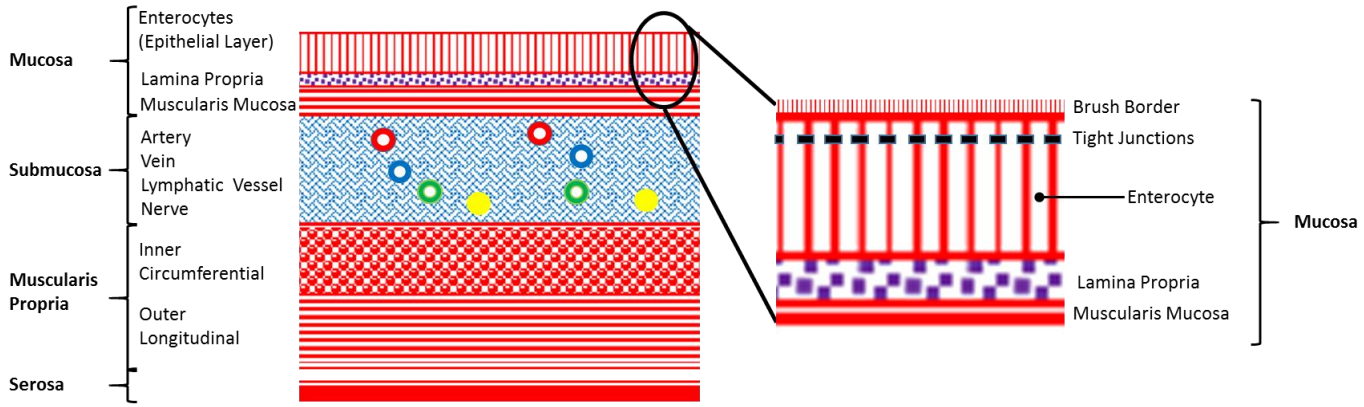

Figure 1: Schematic diagram of the histology of the gastrointestinal (GI) tract at the level of the small bowel. The four cardinal layers are the mucosa, submucosa, muscularis propria and serosa. The luminal-facing mucosa is responsible for digestion, absorption and barrier function. Subsequent layers are the submucosa (vascular, neural and structural support), the muscularis propria comprised of the inner circular and outer longitudinal muscles (food mixing and propulsion, respectively) and the serosa (structural support, lubrication preventing organ adhesion). As the mucosa is in contact with the external environment, preventing the contamination of the sterile inner environment is a vital role of the enterocytes, a monolayer of cells that lines the GI tract. Nutrient channels in the enterocytes are highly specialised and selective for particular molecules (e.g. glucose, amino acids). Material is also blocked from entering the body between cells via apicolateral located tight junctions.

85.1] and sex (12/14 female). Small bowel samples were obtained from colony culls employed as controls for an unrelated experiment. Because of the limited tissue viability, they were processed immediately post-mortem and prepared for use in the shortest amount of time possible. The small bowel was excised via abdominal laparotomy and immediately placed in cold (4°C) phosphate-buffered saline (PBS). The lumen was cleaned with cold PBS via luminal syringe injection. A length of bowel (<55 mm) was then sectioned along its long axis and opened to expose the mucosa. The sample was pinned, mucosa side up, onto an acoustic absorber with a variable number of 25 gauge hypodermic needles (Becton Dickson, USA) to maintain position and prevent excessive tissue curling. To ensure proper orientation of the bowel, i.e. mucosa side up, samples were checked under magnification with improper orientation resulting in rejection because of the amount of manipulation required to correct it. Following the image check, the bowel was placed in a sonication chamber and submerged in PBS at 37°C. Fig. 2 shows the experimental arrangement of tissue, transducer and ancillary equipment

B. *Focused US Transducer*

Focused US transducers were fabricated and characterized using the method described by Stewart et al. [6]. The transducer used was made with PZ54 material with a frequency of 3.31 MHz and radius of curvature R_C = 15 mm. It was driven at 10 V_{pp}, producing an acoustic pressure, p_{AC} = 145.6 kPa, acoustic power, P_{AC} = 76.1 mW, intensity, I = 0.0343 W mm^{-2}, focal beam diameter BD_F = 1.68 mm, and focal beam length, BL_F = 7.9 mm.

C. *Quantum Dots*

Cadmium alloy quantum dots (Sigma-Aldrich, USA) were chosen for both their nanoscale size, Ø: 6 nm, and fluorescent properties for immediate post-sonication check. The CdSe/ZnS quantum dots (qdots) fluoresced at 540 nm or 525 nm and were dissolved in H_2O (1 mg/ml) as supplied. The qdots were further diluted in distilled H_2O to a ratio of 1:10 (qdots:H_2O). The qdot fluid was then loaded into a 60 ml syringe (Becton Dickson, USA) and placed in a syringe driver (Braun, DE) as indicated in Fig. 2. The syringe outlet was carefully situated on the mucosa of the tissue to avoid both too great a tissue-outlet gap and inadvertent forcing of the outlet into the tissue.

D. *Protocol*

The basic sonication protocol consisted of active sonication versus a paired control (qdots only) on the same sample. The sonication arm began with a simultaneous introduction of qdots and signal application to the transducer. The qdots were supplied at variable rates as indicated in Table 1 and halted 30 s after first introduction. Sonication was continued for a further 30 s (60 s total). Control studies were done without sonication where the qdots were introduced for 30s, the supply was halted but the position of the outlet remained for a further 30 s (60 s total).

Initially, tests were done with 1x sonication and 1x control per tissue sample. However, as the tissue preparation technique was refined and improved, tests per sample were increased to 2x sonications and 2x control unless otherwise noted in Table 1. Additionally, the pattern of sonication versus control was altered to account for tissue degradation during the experiments.

Following the introduction of the qdots, with or without US, the tissue sample was washed with 37°C PBS using gentle agitation and gently rinsed with 37°C PBS using a syringe, whilst avoiding epithelial stripping. This was to remove any qdots that might have become trapped in the

Table I. Multitable including serial number (date and sample number), age range (days: 53-115, $\bar{x}$: 85.1), gender (♀: 12,♂: 2) , infusion rate, paired tests per test pattern (s: Sonocation, c: Control) and outcome (12 Success, 3 Fails, 8 Omits).

Date	Sample #	Age (Days)	Gender	Infusion Rate (ml/hr)	Test Pattern	Sonications per sample	Pattern	Outcome (L/R)
2016-06-30	1	55	Female	100	L-R: C1, S1	1	L-R: C1, S1	Success
2016-06-30	2	55	Female	100	L-R: C1, S1	1	L-R: C1, S1	Success
2016-06-30	3	55	Female	100	L-R: C1, S1	1	L-R: C1, S1	Omit
2016-07-19	1	115	Female	100	-	2	-	Omit/Omit
2016-07-19	2	115	Female	100	L-R: C1, S1, C2, S2	2	L-R: C1, S1, C2, S2	Success/Omit
2016-07-19	3	115	Female	100	L-R: C1, S1	1	L-R: C1, S1	Success
2016-07-19	4	115	Female	100	L-R: S1, C1, C2, S2	2	L-R: S1, C1, C2, S2	Fail/Success
2016-07-19	5	115	Female	100	L-R: S1, C1, C2, S2	2	L-R: S1, C1, C2, S2	Omit/Omit
2016-07-19	6	115	Female	100	L-R: C1, S1, C2, S2	2	L-R: C1, S1, C2, S2	Omit/Success
2016-07-22	2	82	Female	75	R-L: C1, S1	1	R-L: C1, S1	Omit
2016-07-22	4	82	Female	50	R-L: C1, S1, S2, C2	2	R-L: C1, S1, S2, C2	Success/Success
2016-07-28	1	66	Female	40	L-R: C1, S1, C2, S2	2	L-R: C1, S1, C2, S2	Fail/Success
2016-07-28	2	53	Male	40	L-R: S1, C1, C2, S2	2	L-R: S1, C1, C2, S2	Success/Success
2016-07-28	3	53	Male	40	L-R: C1, S1, S2, C2	2	L-R: C1, S1, S2, C2	Fail/Success

mucous layer without penetrating the epithelial layer. The tissue was subsequently viewed under fluorescent light (UVGL-58 Handheld UV Lamp, USA) to assess results. Finally, it was fixed in 4% paraformaldehyde (PFA) for later histological studies.

III. Results

Experiments were conducted on 14 mouse models for a total of 23 sonication versus control tests. Of the 23 paired tests, 8 samples (34.8%) were omitted from the final results. This was due to compromised tissue integrity relating to elapsed time, over-manipulation, or extensive tissue curling. Therefore, of the 23 paired tests, 15 (65.2%) were deemed to have acceptable results.

Sonication results were judged qualitatively based on qdot pattern and distribution as well as fluorescent intensity. Based on this assessment results were categorised as success or fail, the latter including equivalent and double negative non-fluorescing results. Fig. 3 presents a selection of results which include examples of success and fail. Of the 15 acceptable results, 12 paired tests were considered successful whilst 3 were rated a fail, giving a success rate of 12 of 15 (80%). Table 1 presents the tabulated results.

IV. Discussion and Future Work

The results of this experiment indicate that focused US can potentially disrupt the protective gut barrier system and allow passage of a marking agent into the tissue. Discrepancies may be explained by the delicate nature of the mouse bowel and the placement of the qdot outlet. Too great a gap between outlet and tissue moves the qdots out of the transducer's focus and result in a double negative result, as demonstrated in Fig. 3C. This type of fails could be mitigated or avoided with incorporation of the qdot outlet into the transducer, thus ensure the agent would exit along the focal axis without risking tissue damage. Conversely, pressing the outlet too hard into the tissue results in mucosal damage, with the qdots injected directly into the tissue. This has the potential to produce the equivalent results shown in Fig. 3D. Reduction of the infusion rate was due to similar results at 100 ml/hr versus 40 ml/hr thus reducing qdot usage and mitigating any inkjet marking that faster rates may have induced.

As mentioned in the Materials and Methods section, the tissue was fixed in 4% PFA for histological analysis. However, it was noticed that samples failed to fluoresce post-fixation (>24 hours). Reasons for this are hypothesised to relate to the fixation process. Either the 4% PFA inactivates the qdots [7] or it causes the qdots to leach out

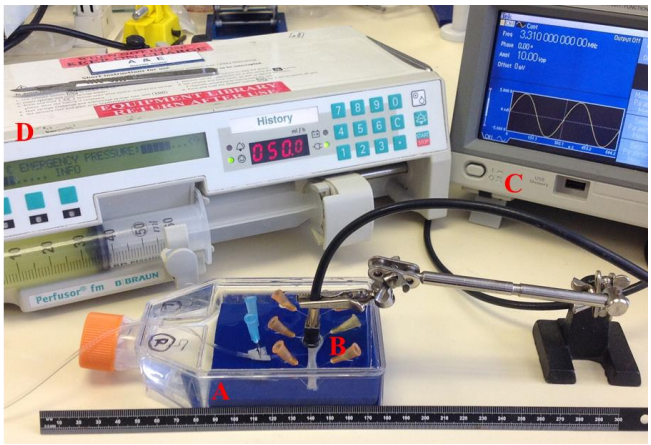

Figure 2. The container, (A), had its base lined with acoustic absorber to which the murine bowel sample (B), was pinned, mucosa upwards. A focused US transducer was positioned above the tissue sample and a signal, amplitude 10 V_{pp}, frequency 3.31 MHz was applied with a signal generator, (C). The qdot solution diluted with distilled H_2O was supplied from a syringe driver (D) at variable rates (ml/hr).

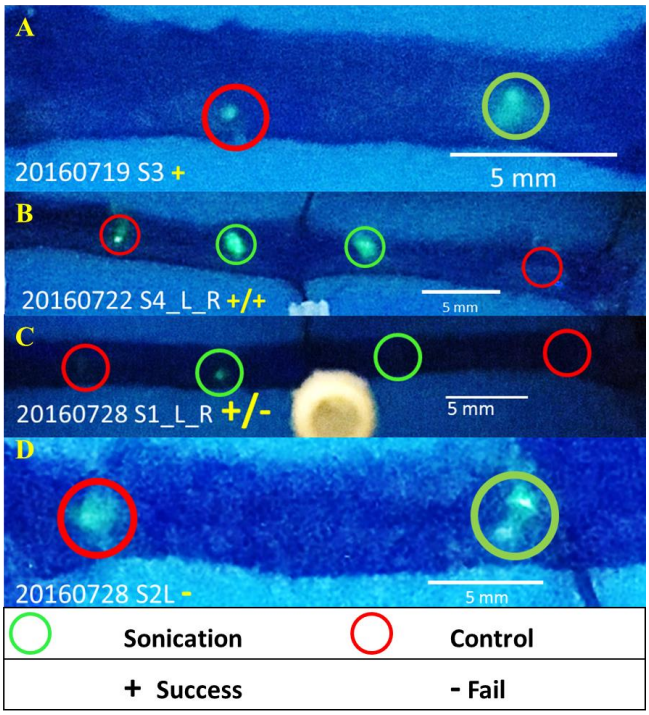

Figure 3: Selected results illustrating examples of success and failure. Fig 3A demonstrates a fluorescing control (L) and sonication result (R). Comparison of the two results yields qualitative differences in the pattern, distribution and intensity of the qdot mark. Fig 3B illustrates a fluorescing control and sonication result (L) with a clear difference in intensity and pattern compared with a non-fluorescing control (R). Fig 3C is an example of a failed test (L) due to the double non-fluorescing results of sonication and control; an additional example of a success (L) is also presented. Fig 3D demonstrates a fail test (R) due to the equivocal nature of the control and sonication results.

into the solution. Of these two reasons, the latter presents the greater concern as it would indicate superficial interaction only, i.e. in the mucous layer. Microscopic examination of unfixed bowel proved unremarkable. The thin nature of the mouse bowel presented difficulty in visually isolating the mucosal layer under ultraviolet light. This was avoided during macroscopic examination by illuminating the tissue on a non-fluorescing surface versus a glass slide.

Determining whether the qdots actually penetrate the enterocytes (intracellular) or pass (paracellular) between them is an area of active investigation. Additionally, porcine models more analogous to the human GI tract are under consideration [8] to improve translational data, reduce technique-dependent issues relating to the small physical scale of the murine model, and ease histological examination. Furthermore, given the toxic nature of cadmium, other biocompatible agents for marking tissue are under review, as well as means of measuring fluorescence quantitatively.

In summary, this work provides an initial indication that that focused US can alter the permeability of the gut barrier and allow passage of a marking agent. Further work is required to elucidate the exact nature of this process.

V. Acknowledgments

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VI. References

[1] Lu KC, Wietz, DW. Small Intestine [Chap 13]. The Washington Manual of Surgery, 4th Edition. Ln: Klingensmith ME, Amos KD, Green DW, Halpin, VJ, Hunt SR 2005, Lippincott, Williams and Wilkins,

[2] Sinnecker H, Krause T, Koelling S, Lautenschläger I, Frey A. The gut wall provides an effective barrier against nanoparticle uptake. Beilstein J Nanotechnol. 2014 Nov 12;5:2092-101.

[3] Delalande, A., Kotopoulis, S., Rovers, T., Pichon, C., & Postema, M.. Sonoporation at a low mechanical index. Bubble Science, 2011, Engineering and Technology, 3(1), 3-12.

[4] Stewart, F., Cox, B., Vorstius, J., Verbeni, A., Qiu, Y. and Cochran, S. Capsule-based ultrasound-mediated targeted gastrointestinal drug delivery. In Ultrasonics Symposium (IUS), 2015 IEEE International (pp. 1-4). IEEE.

[5] Tacker, J. R. and R. U. Anderson. Delivery of antitumor drug to bladder cancer by use of phase transition liposomes and hyperthermia. J Urol 127(6): 1211-1214.

[6] Stewart, F., Wang, G., Newton, I.P., Cox, B.F., Thanou, M., Huang, Z., Nathke, I.S., Cochran, S. An In Vitro Sonication system for Applications in Ultrasound-mediated Targeted Drug Delivery. In Ultrasonics Symposium (IUS), 2016 IEEE International (pp1-4). IEEE.

[7] Zarkowsky D, Lamoreaux L, Chattopadhyay P, Koup RA, Perfetto SP, Roederer M. Heavy metal contaminants can eliminate quantum dot fluorescence. Cytometry A. 2011 Jan;79(1):84-9.

[8] Schantz LD, Laber-Laird K, Bingel SA, Swindle MM. Pigs [Chapter 26]. Essentials of Experimental Surgery: Gastroenterology. H Gregerson, S Lindkaer Jensen, F Moody, M Shokouh-Amiri. 1196, CRC Press.